CHAPTER-2

BASICS OF NMR IMAGING AND SPECTROSCOPY
2.1 Introduction
NMR spectroscopy and imaging a well known non-invasive technique that has wide range applications and the basic principle of NMR has been discussed in this chapter (Orrison et al, 1995; Mitchell, 1999)

2.1.1 A Brief History of NMR
The conception of NMR sprouted after the Pauli’s prediction of nuclear spin in 1924. Later Gorter (1936) attempted to detect the first NMR in bulk matter at 20 MHz in LiF and Al-K alum, which did not worked at low temperature. Isidor Rabi, an american physicist who was awarded the Nobel Prize for Physics in 1944 for his invention of the atomic and molecular beam magnetic resonance method of observing atomic spectra, came across the NMR experiment in the late 1930's but considered it to be an artifact of his apparatus and disregarded its importance.

NMR was first experimentally observed in late 1945, nearly simultaneously by the research groups of Felix Bloch, at Stanford University and Edward Purcell at Harvard University. The first NMR spectra were published in Physical Review in January of 1946. Bloch and Purcell received the Nobel Prize for this invention in 1952 (Physics). Later Packard and Arnold (1951) observed chemical shift phenomenon in ethanol and showed that it is dependent on the solvent. Overhauser (1953) predicted that a small alteration in the electron spin populations would produce a large change in the nuclear spin polarization, which later was named as Overhauser effect. Paul Lauterbur (1973) acquired first NMR based image of water filled capillaries and termed as „zeugmatography“. In 1975, for fourier transformation (FT) spectroscopy, Richard Ernst was awarded Noble prize in 1992 (Chemistry). In 1977, Peter Mansfield discovered Echo Planar Imaging (EPI), which makes image acquisition fast and robust and were awarded Noble Prize (Physiology and Medicine) in 2003.

Presently NMR has played an important role in physics, chemistry and biology for the development of theories and provided experimental platform for spectroscopic and quantum mechanical theories. In the field of chemistry, NMR is used as an analytical tool for reaction studies, for identification and structural elucidation. However, the use of NMR in medicine /biology commenced in 1960. The phenomenon that is studied by NMR, occurs when a static magnetic field is applied to the nuclei of certain atoms (¹H, ¹³C, ³¹P, ¹⁹F etc.), followed by a second oscillating field. Any nuclei with an odd number of protons or neutrons can be measured using
NMR, though $^1$H and $^{13}$C are the most common one. These nuclei exhibit specific rotating electrical charges, or spins properties. There are three steps to get NMR signal.

**First step: Polarization** entails interaction between the set of spin properties and a static magnetic field. This results in a balanced or equilibrium state.

**Second step: Resonance** implies exciting radio frequency (RF) field supplies energy to the spin system, perturbing the previously mentioned equilibrium state. The RF field is applied in short pulses, simultaneously exciting the spin system into transitions between energy states.

**Third step: Relaxation** occurs immediately after the RF pulse, when the spin system returns to the original balanced state. As the relaxation advances, a current is induced in an RF coil, which picks up all the transitions nuclei simultaneously. This current or exponentially decaying signal known as free induction decay (FID), which is then Fourier transformed, resulting in a spectrum.

### 2.2 Physical Basic of NMR

The nucleus accommodates most of the elemental mass of the atom that consists of neutrons and protons each possess a mass of approximately one on the atomic scale. Most nuclear species possess angular momentum or spin, a property first suggested by Pauli in 1924 to explain the fine structure of atomic spectra.

According to the quantum mechanics the angular momentum $\mathbf{p}$ of the atomic nuclei can only have certain discrete values, specified by the magnetic quantum number ($I$).

Magnitude of the angular momentum ($\mathbf{P}$) is given by

$$ P = \hbar \sqrt{I (I+1)} $$

Where $\hbar = h/2\pi$ (h is Plank’s Constant)

$I =$ Magnetic quantum number,

Magnetic quantum number represents spin of the nucleus which has 1 or 1/2 values as follows:

a) $ I = 1 $, for nuclei with even mass number
b) $ I = 0 $, for equal number of neutrons and protons due to the tendency of both these nuclear entity to form pairs in such a way that the individual spin cancel out. Therefore, $^{12}$C and $^{16}$O have zero spin and do not produce NMR signals.

c) $ I = 1/2 $ for nuclei with odd mass numbers. Nuclei of half spin, such as $^1$H, $^{13}$C and $^{31}$P are particularly important in NMR, as these are the nuclei that tend to have the
most appropriate NMR characteristics. Some NMR active nuclei and their properties are listed in Table 2.1.

**Table 2.1: Properties of NMR active nuclei and their properties**

<table>
<thead>
<tr>
<th>Nuclei No</th>
<th>quantum</th>
<th>Resonance frequency (MHz)/ T</th>
<th>Natural abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>1/2</td>
<td>42.6</td>
<td>99.98%</td>
</tr>
<tr>
<td>$^2$D</td>
<td>1</td>
<td>6.5</td>
<td>0.0156%</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>1/2</td>
<td>10.7</td>
<td>1.1%</td>
</tr>
<tr>
<td>$^{15}$N</td>
<td>1/2</td>
<td>4.3</td>
<td>0.36%</td>
</tr>
<tr>
<td>$^{19}$F</td>
<td>1/2</td>
<td>4.4</td>
<td>100.0%</td>
</tr>
<tr>
<td>$^{23}$Na</td>
<td>3/2</td>
<td>11.3</td>
<td>100.0%</td>
</tr>
<tr>
<td>$^{31}$P</td>
<td>1/2</td>
<td>17.2</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

Angular momentum is a vector quantity, i.e. it is specified by both its magnitude and direction. In order to specify the direction of the angular momentum, it is necessary to introduce a second quantum number i.e. angular quantum number (m). It is found that the angular momentum vector can only have certain discrete orientations with respect to any given direction. The component $P_z$ of the angular momentum along the Z-direction is given by

$$P_z = m \hbar \quad (2)$$

Angular quantum number (m) may have any of the $2I+1$ values, and thus for a nucleus of spin $1/2$, $m$ can be $+1/2$ or $-1/2$. Therefore, for such a nucleus the two spins are shown in Fig. 2.1

$$P_z = \pm (1/2) \hbar \quad (3)$$
Fig. 2.1 The allowed orientations of the angular momentum vector of a nucleus of spin 1/2 which is specified by the quantum number (m =1/2 and -1/2) and describe two cones.

2.2.1 Nuclei in a Static Magnetic Field
Most of the nuclei relevant to biological NMR, such as $^1$H possess a spin quantum number I =1/2 and therefore according to quantum mechanics, it can adopt two different orientations relative to an arbitrary axis. In the absence of magnetic field (Bo) these spins are randomly oriented with respect to each other; however when subjected to a magnetic field (Bo) the spins align themselves with and against the direction of Bo corresponding to high and low energy spin states as shown in Fig. 2.2.

Fig. 2.2: Nuclei of spin orientation when B = 0; B = Bo two possible spin states

Boltzmann statistic show that, for protons at room temperature in the high magnetic field, the difference between lower energy state versus higher energy state is of the order of one
in a million and amazingly that resultant nuclear magnetism is still detectable. At room temperature, the number of spins in the lower energy state, $N_+$, slightly outnumbers the number in the higher energy state, $N_-$. So according to the Boltzmann equation

$$\frac{N_-}{N_+} = e^{-\Delta E/kT} \quad (4)$$

Where

$\Delta E =$ Energy between the spin states

$K=1.3805 \times 10^{-23}$ J/Kelvin (Boltzmann's constant),

$T =$ Temperature in Kelvin

The resultant nuclear magnetism is proportional to the applied magnetic field (Fig. 2.3).

**Fig. 2.3:** In the presence of magnetic field ($B_0$), two energy levels are established. The energy and the population difference increase with the applied field $B_0$.

### 2.2.2 Magnetic Moment of Nucleus

Since the nuclei bear electrical charge, their spinning produces a magnetic moment ($\mu$), expressing the strength and direction of the magnetic field surrounding the nucleus. The field produced by these magnetic dipoles is analogous to those of a microscopic bar magnets shown in Fig. 2.4. The magnetic moment of the nucleus is closely related to its angular momentum and its direction is same as that of the angular momentum. The magnitude of $\mu$ is given by

$$\mu = \gamma p \quad (5)$$
Where $\gamma = \text{Gyro-magnetic ratio (constant)}$ of the nucleus.

Fig. 2.4 The spinning nuclei with electrical charge on it behave like a bar magnet.

The torque generated by $B_o$ on the magnetization (i.e. magnetic moment per unit volume), results in expression of $M$ about $B_o$ at a frequency

$$v = \gamma B_o / 2\pi$$

(6)

The angular frequency of precession, is the Larmor frequency and is given by

$$\omega = \gamma B_o$$

(7)

2.2.3 Longitudinal and Transverse Magnetization

The random phase of the precessing magnetic moments of nucleus create a macroscopic net magnetization $M_{\text{net}}$, a vector quantity having longitudinal and transverse components that is ultimately responsible for the induction of NMR signal (Fig. 2.5).

Fig. 2.5: Net magnetization $M_{\text{net}}$ (longitudinal magnetization) and $M_{xy}$ (transverse magnetization)

The applied RF field of amplitude $B_1$, acting in a perpendicular to the main magnetic field, rotates the magnetization $M_{\text{net}}$ away from its equilibrium state, similar to the individual magnetic moments sense the force of the external field, forcing the
magnetization to rotate about it (Fig. 2.6). The time constant which describes how $M_Z$ returns to its equilibrium value is called the spin lattice relaxation time ($T_1$).

**Fig. 2.6:** In the presence of Radio Frequency (RF) field $B_1$ that is rotating synchronously with the X axis, the magnetization ($M_{\text{net}}$) in the rotating frames rotates about the X axis by an angle $\alpha$.

The motion of these magnetization vectors can be described using a frame of reference with co-ordinate system in which X and Y axes rotate in synchrony with the $B_1$ field. By contrast, in a static frame of reference, the tip of the magnetization vector would describe a spiral path from its original orientation (Z-axis) down to XY plane (Fig. 2.7). The time constant this describes the return to equilibrium of the transverse magnetization (XY plane), is called the spin-spin relaxation time, ($T_2$).
Fig. 2.7: The spiral nutating path of magnetization $M_{\text{Net}}$ after RF excitation. The RF field of amplitude $B_1$ is applied along the direction (x) perpendicular to the main magnetic field.

Once the applied RF (excitation) pulse is removed, the magnetization is subjected only to the static magnetic field ($B_0$) and hence starts precessing about it. The induced voltage is detected by placing a RF receiver coil with its axis oriented along the Y axis of the static coordinate system (Fig. 2.8) and is given as

$$V \propto M_{XY} \cos \omega$$ \hspace{1cm} (8)

Fig. 2.8: The RF receiver coil placed along the Y-axis detects the magnetization oscillating around the transverse plane (xy) after cessation of the RF excitation.

Where $M_{xy}$ is the initial transverse magnetization following a 90° rotation by the $B_1$ field (90° RF pulse) and "t" is the time interval between the 90° rotation and the voltage measurement. The signal induced is a cosine of the amplitude proportional to the magnetization and frequency equal to the Larmor frequency. However, the transverse magnetization $M_{xy}$ does not last indefinitely but the equilibrium magnetization recovers exponentially through longitudinal relaxation time constant $T_1$ and transverse relaxation time constant $T_2$. Thus, the amplitude of the detected voltage $V$ can be described more accurately as:

$$V \propto M_{xy} e^{-t/T_2} \cos \omega t$$ \hspace{1cm} (9)
2.2.4 Free Induction Decay

The decaying signal after 90° RF pulse is known as “free induction decay” or FID (Fig. 2.9). Further processing with fourier transformation (FT) of this detected signal provides information about the object under study either in the form of frequency spectrum of the molecules or image.

![Free Induction Decay](image)

**Fig. 2.9:** The free induction decay (FID) signals created as a result of oscillation (damped) of the net magnetization in the transverse plane. Three different FIDs of compound with increasing complexity.

2.3 Nuclear Magnetic Resonance Spectroscopy

Only those nuclei that have magnetic properties give rise to NMR signals. The resonance frequency of these nuclei is directly proportional to the local magnetic field experienced nucleus. The nuclei of different elements (e.g. $^1$H, $^{13}$C, $^{31}$P etc) and compounds resonate at different frequencies after RF pulse induction in magnetic field (Bo), because of its different nuclear properties.

2.3.1 Chemical Shift ($\delta$)

The NMR spectroscopy measurements are based on the chemical shift which is associated with the field variation due to electrons and adjacent nuclei. The chemical shift ables to differentiate protons of water from amino acids, sugars, membrane metabolites etc in the NMR spectrum due to different chemical environment. This is because each $^1$H nucleus is surrounded by other nuclei and electrons that induce electronic current in atoms and molecules. The electronic current in turn produces a further magnetic field which is proportional to Bo. In fact it is electrons in the chemical bonds that are most significant in affecting the magnetic field experienced by a nucleus due to its large magnetic moment. The total effective magnetic field ($B_{\text{eff}}$) at the nucleus can therefore be expressed as
\[ B_{\text{eff}} = B_0 (1 - \sigma) \]  

Where \( \sigma \) is known as the shielding constant, which represents the contribution of the small secondary field generated by the electrons, and is in the range of \( 10^{-6} \) to \( 10^3 \). Hence, according to equation (7), the resonance frequency of the nuclei is

\[ v = \left( \frac{\gamma}{2\pi} \right) B_0 (1 - \sigma) \]  

The magnitude of \( \sigma \) depends on the electronic environment of the nucleus. Even though, the shielding parameters \( \sigma \) is constant, the measured chemical shifts increase linearly with the field strength. Therefore, to compare chemical shifts measures at different field strengths, the chemical shifts values are usually expressed in parts-per-million (ppm) of the resonance frequency, measured relative to the frequency of a reference compound (\( v_{\text{ref}} \))

\[
\text{Chemical shift (}\delta\text{)} = \left[ (v_{\text{ref}} - v) \times 10^6 \right] / v_{\text{ref}}
\]

2.3.2 Signal Intensity
The quantitative information of the concentration of chemical compounds can also be obtained from the NMR spectrum by calculating the spectrum intensity or the area under the peak. The area under the peak is proportional to the number of nuclei that contribute to that chemical compound. However, the effect of spin lattice relaxation (T1) and spin-spin relaxation (T2) could also influence the signal intensities. These relaxation effects could be used for selective detection or suppression of specific signals.

2.3.3 Spin-Spin Coupling (Multiplicity)
NMR spectroscopy also helps in analysis of chemical compound and its molecular structure by providing magnetic interaction between adjacent nuclei, also known as scalar or \( J \)-coupling. The coupling causes the signal of each nucleus into characteristic intensities and spacing which depend upon the number and type of spin coupling with the nucleus of interest. Certain factors regulate the splitting pattern are:

1. The magnitude of the splitting is independent of the applied magnetic field \( B_0 \).
2. The spacing between peaks is also a characteristic feature of that particular compound such as lactate quartet (\( J \)-coupling constant= 6.933 Hz) and alanine quartet (7.234 Hz).
3. No splitting (singlet) between identical protons or protons having similar environment.

4. A proton coupled to n number of identical protons gives rise to (n+1) lines.

5. The splitting pattern of the lines given by binomial distribution i.e. the CH₃ proton coupled to CH Fig. 2.10) give rise to two lines (doublet) with equal intensities while CH group coupled to CH₃ give rise to four lines (quartet) with relative intensities 1:3:3:1.

Fig. 2.10: ¹H NMR spectra of lactate and splitting pattern of CH₃ and CH peaks.

2.4 Sequences Employed in Biological ¹H NMR Spectroscopy

Most of the sequences used to study for biological sample are tailed together with presaturation (pr) pulses. In presaturation”, solvent signal is irradiated selectively with a weak RF field (60-70 dB on spectrometers) during the 1-2 second relaxation delay of a 1D experiments. The selection of pulse sequences depends on nature of biological sample and information required from that sample. Few sequences which are largely employed in biological sample analysis are as follows:

2.4.1 NOESY

Stands for nuclear overhauser enhanced spectroscopy and the sequence is based on nuclear overhauser principle that small alteration in the electron spin populations would produce a large change in the nuclear spin polarization. The pulse sequence (RD-90°-t-
90°-tm-90°-acq) shows three alternative 90° pulses with relaxation delay (RD) of 2.0 seconds and an acquisition time (acq) of 2.5 seconds. NOESYPR sequence is used to acquire urine spectrum, which enhances the detection of metabolites from urine sample.

Fig. 2.11: A block diagram of NOESY pulse sequence.

2.4.2 Carr Purcell Meiboom Gill (CPMG)
This sequence is initially developed by Carr and Purcell and further modified by Meiboom and Gill. CPMG sequence (RD-90°-(s-180°-s) n-acq) is spin echo sequence which are used for the measurement of T2 relaxation constant. This sequence is used to acquire serum NMR spectrum because the pulse sequence with a total spin echo of 200 ms used to attenuate broad signals from proteins and lipoproteins of serum. This makes small metabolites could easily be detectable from serum NMR spectrum.

Fig. 2.12: A block diagram of CPMG pulse sequence

2.4.3 ZGPR
ZG stands for Zero and Go. Zero infers zero memory that is previous memory washed out and G stands for to run the experiment. ZGPR is a single pulse sequence (RD-90°
–acq) with low power presaturation pulse followed by high power 90° pulse. It is a basic sequence (ZG) to select solvent (water) peak followed by irradiation. The ZGPR sequence was used to analyze the aqueous tissue extract.

![Fig. 2.13: A block diagram of CPMG pulse sequence](image)

2.4.4 2D- NMR Spectroscopy

Two dimensional NMR spectroscopy can be useful for increasing signal dispersion and for elucidating the connectivity between signals, thereby enhancing the information content, helping to identify the biochemical substances. 2D- NMR spectroscopy is performed for identification and conformation of metabolites. The „basic” 2D spectrum would involve repeating a multiple pulse 1D sequence with a systematic variation of the delay time \( t_D \), and then plotting everything stacked. Basically 2D spectroscopy consists of preparation, evolution, mixing and acquisition phases. The first perturbation of the system (pulse) is called preparation of the spin system then variable delay time is renamed as evolution time \( t_1 \) followed with a mixing event, in which information from one dimension to the second dimension of the spin system are transferred and finally the acquisition time \( t_2 \). A simple 2D experiments such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) provides $^1$H-$^1$H spin-spin coupling connectivity, giving information regarding which hydrogen atom in the molecule are in close vicinity can be identified by cross peaks. Use of other type of nuclei, such as $^{13}$C or $^{15}$N, or $^{31}$P can be performed in heteronuclear correlation experiments (HSQC, HMBC). The heteronuclear experiment employ the inverse detection methodology, where the lower sensitivity or less abundant nucleus spectrum (such as $^{13}$C) is detected indirectly using the more sensitive/ abundant nucleus ($^1$H) by making use of spin-spin interaction such as the one bond $^{13}$C-$^1$H spin-spin coupling between the nuclei. This results both $^1$H and $^{13}$C NMR chemical shifts of CH, CH$_2$ and CH$_3$ groups, useful for identification purposes.
2.5 Chemometrics in metabolomics

NMR spectroscopy of the biofluids or tissue extracts contain hundreds to thousands of signals which make the interpretation of data difficult, in order to resolve this issue pattern recognition (PR) and multivariate statistical analysis is required. Principal Component Analysis (PCA) is most commonly and easily applied pattern recognition (PR) technique used for metabolomics data analysis. PCA being unsupervised in nature does not give information regarding the treatment related variation that is necessary for interpretation of biological results. PLS is one such supervised method which helps in predicting the final consequences of any kind of treatment in terms of the metabolites which got altered. Before doing the PR analysis, there are few preprocessing step between raw spectra and data analysis, which needs to carry out for ease and improved data analysis.

2.5.1 Data preprocessing step in NMR based metabolomics

**Baseline correction:** The first step of data preprocessing is the removal of any baseline distortion if present. Baseline correction is required for correct statistical analysis as well as for precise quantification of metabolite. These distortions can be corrected in many different ways; usually an automated baseline correction is applied. After baseline correction the region of the regions of the spectrum which might cause interference in the data analysis are removed. In metabolomics of biofluids water signal dominates the spectrum between 4.7 ppm and 5.0 ppm which needs to be removed. Apart from this in case of urine spectra, the signal of urea which is very close to water region is also removed. Moreover it is not
quantitative, because protons from urea exchange with water and other exchangeable protons and thus the peak intensity varies with quality of water suppression as well as with pH.

**Alignment:** It includes presence of peak shifts between different spectra, which might be due to instrumental factors, changes in pH and temperature, changes in salt concentration, overall dilution and relative concentration of specific ions. Therefore alignment is an essential step in preprocessing. There are various kinds of alignment processes, but spectral referencing is used in this thesis, which includes alignments of the peaks with respect to some reference standard (TSP in this case) to 0 ppm.

**Binning:** An NMR spectrum, after removal of water and urea signals still contains many variables making the NMR spectrum to be complex. Therefore in order to reduce the data dimensionality binning also called bucketing is commonly used. In binning the whole spectrum is divided into small segments also called bins or buckets and the total area within each bin is calculated to represent the whole spectrum. Due to this some minor peak shifts can be removed during binning. There are many type of spectral binning but most commonly used binning is equidistant binning of 0.04 ppm which is mainly used in this thesis.

**Normalization and scaling:** Normalization is commonly used to remove or minimize the effect of variable dilution of the samples. Row operation is generally described as normalization for example dividing each feature value by some value such as their sum or mean. Column operation is generally referred as scaling. Scaling removes the variation in the present in the concentration of the metabolites which can influence the data analysis. A number of scaling procedure are available but mean centering, autoscaling, pareto scaling are commonly used. Mean centering adjusts for differences between the low and high concentrated metabolites by converting all values to vary around zero instead of around the mean of the metabolite level. Mean centering is commonly used with other scaling methods. Autoscaling scales all metabolites to unit variance and therefore the data is analyzed on the correlations basis instead of covariances. Autoscaling increases the influence of noisy variables. Due to this reason pareto scaling is commonly used for metabolomic data. Pareto scaling uses the square root of standard deviation as scaling factor instead of the standard deviation. This
scaling method stays closer to real measurement but it is sensitive to large changes in the data.

2.5.2 Principle component analysis (PCA)
Principal component analysis (PCA) was first introduced in statistics by Pearson (1901) with a geometric interpretation of „lines and planes of closest fit to systems of point in space“, and Hotelling (1933) further developed PCA to its present stage i.e. to describe the variation in a set of multivariate data in terms of a set of uncorrelated variables. PCA can be generally described as a method that reveals the internal structure of a data set in a way which best explains the variance in the data.

Mathematically a PCA model can be written as:

\[ X = T \cdot P^T + E \]

Where \( X \) is the data matrix representing samples and variables decomposed into a score matrix (\( T \)) and a transposed loading matrix (\( P^T \)). The E matrix contains the residuals, the part of the data not „explained“ by the principal component model. In this way, the score and loading matrix contains the systematic variation with respect to samples and variables, leaving the unsystematic variation in the residual (Wold, 1987). Each combination of \( t \cdot p \cdot T \) (where, \( TP^T = t_1p_1^T + t_2p_2^T + \ldots \)) is called a Principal Component (PC). The first PC (i.e. \( t_1p_1^T \)) describes the maximum (the largest) variation in the actual data; the second PC describes the second largest variation and so on, each PC being mutually orthogonal. The score (\( T \)) represents the observations (i.e. row) in the X matrix and loading represents the variables (i.e. column) in the X matrix. The projection of the actual sample in the model provides a score plot, where the relation among the observations or samples in the model plane is visualized i.e. any grouping, trend or any outliers among the observations. PCA offers a reduced dimensional model that summarises the major variation in the data into few axes, and in this way, systematic variation is captured in a model that can be used to quickly visualise which samples in the data set are similar or dissimilar to each other. From this, possible spectral loadings causing any treatment-related separation may be identified. One important feature of PCA model is that the directions in the score plot correspond to directions in the loading plot, and vice versa. This will help in understanding the relationship between the object
grouping in the low dimensional model planes and the variable(s) that is causing the segregation of the relevant groups.

Fig. 2.15 illustrates the geometric explanation of the above discussion.

**Fig. 2.15:** The geometric interpretation of PCA modeling. A- The samples (rows) and variables (columns) in the original data matrix are plotted in the multidimensional space. B- The PCs are computed so that the 1st PC explains the maximum variation in the samples, the 2nd PC explains the second largest and so on. The PCs are mutually orthogonal. C - Further, each sample from the multidimensional space are projected onto the scores plane t1-t2 giving rise to the score plot. This clearly explains the grouping in the sample set. D- The variables are projected on the loadings plane to provide the loadings plot.

2.5.3 Partial least squares (PLS)

Partial least square is a statistical method that uses same principal as that of PCA but it also uses second piece of information i.e. labeled set of class identifies. PLS is used to find the fundamental relation between two matrices X and Y. Among them the X- is the matrices comprising of descriptor variable (e.g. NMR spectra) and Y is the response variable / biomarker containing of quantitative or qualitative (such as a class belonging, treatment of the samples) variables. This enables the establishment of a linear model that can predict Y from the measured spectra in X. Like PCA, PLS regression generates a linear model of the data, but where PCA models the major variation in the data itself,
PLS derives a model that describes the correlation between the X variables and a feature (Y variable) of interest (Keun, 2006).

The general underlying model of multivariate PLS is

\[ X = TP^T + E \]
\[ Y = UQ^T + F \]

Where X is a matrix of predictors, Y is a matrix of responses; T and U are the scores; P and Q are loading matrices; and matrices E and F represent the model residual. The decompositions of X and Y are made so as to maximise the covariance of T and U.

**2.6 Magnetic Resonance Imaging**

Magnetic Resonance Imaging (MRI) is an imaging modality that creates images using the principle of NMR. In 1973 Lauterbur showed image of water filled capillaries can be generated and can be reconstructed by superimposing linear field gradient on the main magnetic field. MRI can generate section of images from any arbitrary plane or direction.

**2.6.1 Magnetic Field Gradient**

The gradient designates the dynamic alteration of magnetic field along one particular direction. Magnetic field is directly proportional to the larmour frequency (\(\omega\)) of protons. This makes variation in frequency by using varying magnetic field along particular direction.

\[ \omega_i = \gamma (B_0 + G) \]

Where \(\omega_i\) = Variable frequency
\(\gamma\) = Gyro magnetic ratio

\(B_0\) = Magnetic Field

G= Gradient along i direction (i = x, y, z directions)

To generate magnetic field gradiaent in z- direction (main magnetic field) a pair of gradient coil placed in opposite direction known as “Maxwell pair” through which current flows in opposite direction. This gradient coil adds and subtract in the main magnetic field (\(B_0\)) in opposite direction i.e. creating a magnetic field gradient along z-direction. Another type of coil known as “Golay coils” are placed in XY plane and
generate gradient along XY axis (Fig. 2.16). The gradient generated by these coils can produce image in certain plane of the imaging object.

![Diagram](image)

**Fig. 2.16:** Magnetic field (Bz) add and substract along Z- direction Gradient

### 2.6.2 Frequency Encoding
The point in the center of the magnet where (x,y,z) =0,0,0 is called the isocenter of the magnet. The magnetic field at the isocenter of the magnet is $B_0$ and the resonant frequency is $\omega_0$. If an object is placed at isocentre of the magnet then each region in x, y and z axis experience different magnetic fields. The result is an NMR spectrum with more than one signal due to different magnetic field. The amplitude of the signal is proportional to the number of spins in a plane perpendicular to the gradient. This procedure is called frequency encoding and causes the resonance frequency to be proportional to the position of the spin. This principle forms the basis behind all magnetic resonance imaging. To demonstrate how an image might be generated from the NMR spectra. Three anatomical planes which are orthogonal in direction usually imaged viz. axial, coronal and sagittal plane (Fig. 2.17). These planes are also known as slice selection plane.
2.6.3 Slice Selection
Slice selection in MRI is the selection of spins in a plane through the object. Slice selection is achieved by applying a one-dimensional, linear magnetic field gradient during the period that the RF pulse is applied. Multislice imaging applies same slice selection gradient for each slice with a different excitation pulse. A frequency encoding gradient is turned on once the slice selection pulse is turned off. The frequency encoding gradient is composed in x and y axis direction.

2.6.4 Phase Encoding
The phase encoding gradient contributes a specific phase angle to a transverse magnetization vector i.e. in XY plane. The specific phase angle depends on the location of the transverse magnetization vector. The transverse magnetization vector from each spin has been rotated to a position along the X axis. The three vectors have the same chemical shift and hence in a uniform magnetic field they will possess the same larmor frequency for example f1 in the Fig. 2.18. While the phase encoding gradient is on, each transverse magnetization vector has its own unique Larmor frequency. Phase encoding gradient is pulsed for a short duration between the time of initial RF pulse and the appearance of MR signal.
**Fig. 2.18**: Spatial encoding of a two-dimensional slice typically employs constant frequency encoding gradient in one direction and phase encoding gradient with a different amplitude in another direction. This results in a unique combination of frequency and phase for each pixel in a slice.

2.6.5 Image Reconstruction
The slice selection gradient is always applied perpendicular to the slice plane. The phase encoding gradient is applied along one of the sides of the image plane. The frequency encoding gradient is applied along the remaining edge of the image plane. Following table indicates the possible combination of the slice, phase, and frequency encoding gradient.

**Table 2.2**: Selection of slice plane and different gradient direction for image acquisition.

<table>
<thead>
<tr>
<th>GRADIENTS</th>
<th>Slice Plane</th>
<th>Slice</th>
<th>Phase</th>
<th>Frequency</th>
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</thead>
<tbody>
<tr>
<td>XY (Axial)</td>
<td>Z</td>
<td>X or Y</td>
<td>Y or X</td>
<td></td>
</tr>
<tr>
<td>XZ (Coronal)</td>
<td>Y</td>
<td>X or Z</td>
<td>Z or X</td>
<td></td>
</tr>
<tr>
<td>YZ (sagittal)</td>
<td>X</td>
<td>Y or Z</td>
<td>Z or Y</td>
<td></td>
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MR signal is always detected in frequency encoding direction. This provides one of the visual dimension required for producing a two dimensional image. The second visual dimension is phase encoding in which phase of the precessing nuclei is used to construct
a 2D- raw data matrix. The matrix consists of time domain signal yield a 2D wave pattern with interference fringes. The matrix is converted to final image by a two step fourier transformation (FT) process. First FT in frequency encoding direction and second in phase encoding direction to convert the time domain data into frequency domain that is final image (Fig. 2.19).

**Fig. 2.19:** A 2-dimensional Fourier transform mathematically converts from spatial frequency to reconstructed MR images.

### 2.7 Factors Affecting Image Quality

The usefulness of constructed image is based upon adequate spatial resolution, low noise level, high signal to noise ratio (SNR), optimum contrast and no artifacts.

#### 2.7.1 Signal to Noise Ratio (SNR)

SNR increases linearly with increasing the field strength of the magnet (B₀) and number of scans. The MR signal is coherent so that the signal will increase with increasing number of scans while noise is incoherent and increases only as square root of the number of scans. The frequency spectrum of the noise is uniform over the complete spectrum and this type of noise is known as white noise.

#### 2.7.2 Data Sampling Rate and Receiver Bandwidth

MR signal is digitized or sampled at an equal interval of time known as dwell time.
Dwell time = \( \frac{1}{2} \times \text{Spectral width (S.W)} \)

Number of data point acquired = \( 2 \times \text{S.W} \times \text{Acquisition time (AT)} \)

Therefore sampling rate should be equal or larger than twice the largest frequency. This is called Nyquist frequency.

Nyquist frequency = \( \frac{1}{\text{Dwell time}} = 2 \times \text{Largest sampling frequency (Fmax)} \)

If Nyquist criterion is not satisfied under sampling of the data that is signal corresponding to frequencies larger than Fmax/2 will appear folded over into the image as an artifact known as Nyquist ghost.

### 2.7.3 Image Resolution

Resolution is a measure of image quality. The resolution is a function of many variables; \( T_2 \), SNR, sampling rate, slice thickness, and image matrix size, to name a few. The relationship between resolution, field of view (FOV), and number of data points, \( N \), across an image is quit understandable. However one can never resolve two features located less than FOV/\( N \), or a pixel apart, in this case increasing the number of data points will decrease the pixel size, but not improve the resolution. Even with a noiseless image and optimal contrast, you may not be able to resolve two features the size of a pixel because field inhomogeneity (\( T_2^* \)) comes into play. Image resolution also depends upon scan time, relaxation time, number of excitation, phase encoding steps and the relation is as follows:

Scan time \( \sim \) Relaxation time \( \times \) No. of phase encoding steps \( \times \) No. of excitation.

### 2.7.4 K-Space

K-space often refers to the temporary image space, usually a matrix, in which data from digitized MR signals are stored during data acquisition. K is used to denote the spatial frequency of a 2D space in which the FT operation is performed. The k space component is Kx and Ky where x and y represents frequency variables (unit is cycle/cm). Every sampling point of the raw data matrix corresponds to a column position and every scan to a line. Each point in k space contains a part of information regarding image rather directly represent point. Low spatial frequency encoded in centre of k-space i.e. K= 0 and this determines gross image feature and image contrast. Whereas high spatial frequency present at the periphery of k-space coded for edge information and spatial resolution (Fig. 2.20).
2.8 Intrinsic Contrast Mechanisms

The versatility of MRI is achieved by exploiting contrast mechanisms to generate good images. The contrast mechanisms in MR imaging are based on tissue-specific parameters, utilized with appropriate pulse sequence choice, sometimes with the application of a contrast agent. The intrinsic contrast mechanisms are based on T1, T2/T2* and proton density (PD).

2.8.1 T1 Weighted Imaging

T1-weighted imaging is performed particularly differentiating fat from water. In T1 weighted images water is dark and fat is bright. This type of imaging performed by gradient echo (GRE) sequence utilizes short TE and short TR. A T1 reducing gadolinium contrast agent is also commonly used, with a T1 scan being collected before...
and after administration of contrast agent to compare the difference. In the brain, T1-weighted scans provide good gray matter/white matter contrast.

**Fig. 2.21:** The maximum T1 difference in fat and water at short TR

2.8.2 **T2 Weighted Imaging**
T2-weighted imaging performed by spin echo sequences utilizing long echo time (TE) and long TR. T2-weighted imaging could also differentiate fat from water but in this case fat shows darker, and water lighter. For example, in the case of cerebral and spinal study, the CSF (cerebrospinal fluid) will be lighter in T2-weighted images. These scans are therefore particularly well suited to imaging edema, with. Because the spin echo sequence is less susceptible to magnetic field inhomogeneities, T2-weighted images are extensively utilized in clinical studies.

**Fig. 2.22:** The maximum T2 difference in fat and water at Long TR and long TE.

2.8.3 **Susceptibility (T2*) Imaging**
T2* ("T2 star") weighted imaging use a gradient echo (GRE) sequence, with long TE and long TR. The GRE sequence does not have the extra refocusing pulse (180°) so it
experiences $T_2$ decay to a greater extent. This additional decay of signal considered as $T2^*$ or susceptibility effect. The $T2^*$ is more prone to susceptibility losses at air/ tissue boundaries, but can increase contrast for certain types of tissue, such as venous blood i.e. in perfusion imaging.

![T2* Decay](image)

**Fig. 2.23:** Schematic diagram of $T2^*$ in a free induction decay signal.

### 2.8.4 Proton Density (PD) Imaging
As the name implies, tissue contrast is provided based on the sheer number of protons within a voxel, which differs across tissue types. PD imaging is one of the simplest forms of MR contrast. The net magnetization of each voxel (volume element) is composed of individual spins within that voxel. To maximise the proton density weighting of an image, the effects of $T1$ and $T2$ are minimized by using long TR and short TE. To enhance the weighting of different type of image contrast long and short TR and TE could be selected, as we can see in the Table 2.3.

<table>
<thead>
<tr>
<th>TE</th>
<th>SHORT</th>
<th>LONG</th>
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<tr>
<td>TR</td>
<td></td>
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<tr>
<td>LONG</td>
<td>Proton Density</td>
<td>$T2^*$ Weighted</td>
</tr>
<tr>
<td>SHORT</td>
<td>$T1$ Weighted</td>
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### Table 2.3: Type of Image contrast on the basis of long and short TR and TE

### 2.8.5 Diffusion Weighted (DW) Imaging
Diffusion means incoherent molecular motions by thermal energy, or so-called Brownian motion. The DW imaging provides information of various parameters that help to characterize tissue composition. DW imaging allows for noninvasive
characterization of tissues on the basis of diffusion of water molecules, physical properties of tissue constituents and tissue microstructure.

Magnetic field gradient introduces linear magnetic field inhomogeneity by gradient orientation (+,− gradient direction), strength (G) changing it’s duration (δ) and polarity, however MR can detect only the signal loss (imperfect rephrasing, Fig. 2.24). There are three important factors that contribute to amount of signal loss as following

I. First, faster the diffusion (high polarity) means most protons changing their location resulting in signal loss.

II. Second, the longer the time separation (δ) between the two gradient pulse (∆) leads to more signal loss.

III. Third, the phase gradation (G and δ) responsible for dephasing and rephrasing, resulting in signal loss.

The slope of signal decay (diffusion constant) could be calculated with the application of different b-value and the formula given in Fig. 2.24.
Fig. 2.24: Mathematical description of the signal loss due to the diffusion process with a pair of opposite polarity gradients and with different b values, the diffusion constant can be calculated from the slope (steeper the slope, the larger the diffusion constant) Blue color indicates an area with high diffusion constant and red, with low diffusion constant. A schematic diffusion weighted images with different b values and a calculated map of the diffusion constant (after arrow) is called the apparent diffusion constant (ADC) map (Adapted from Mori S, 2002).
In diffusion weighted (DW) image, brain region with high diffusion constant (ventricles) become darker, while region with low diffusion constant become brighter. The inverse contrast of DW image inferred as apparent diffusion coefficient (ADC) map
also known as mean diffusitivity (MD) map. In biologic tissue, there is a high probability that water molecules interact with structures such as cell membranes, macromolecules that reduce or impede its motion. Water exchange, between intracellular and extracellular compartments, as well as the shape of extracellular space and tissue cellularity, affects diffusion. In this case, the term apparent diffusion coefficient (ADC) represents the measured diffusion constants and is commonly reported in cm$^2$/s or mm$^2$/s.

**Isotropy and anisotropy**

Isotropy means uniformity in all directions. A drop of ink placed in the middle of a sphere filled with water spreads over the entire volume, with no directional preference. If the same experiment is repeated in a sphere filled with uniform gel the restriction is increased as compared with free water, but is still isotropic, as the restriction is the same in all directions. Anisotropy implies that the property changes with the direction. If a bundle of wheat straw with the fibers parallel to each other is placed inside a glass of water, the ink will face severe restriction in the direction perpendicular to the fibers and facilitated along the fibers. This bundle is highly anisotropic.

In DTI, we use this anisotropy to estimate the axonal organization of the brain. Namely, water should move more easily along the axonal bundles rather than perpendicular to these bundles because there are fewer obstacles to prevent movement along the fibers (Stejskal, 1965). When we characterize an-isotropic diffusion, it provides us with an entirely new image contrast, which is based on structural orientation (Chenevert et al., 1990; Moseley et al., 1990; Turner et al., 1990).

The direction of water diffusion in x, y and z- direction on pixel to pixel basis, delineates the directionality of nerve fiber, makes the foundation of diffusion tensor imaging (DTI). The orientations of different fiber are estimated from three independent diffusion measurements along the X, Y, and Z axes. However, these measurements are not enough because fiber orientation is not always along one of the three axes but in reality, they are almost always oblique to the axes. In order to accurately find the orientation with the largest ADC (the movement of water molecules in a particular direction which determines the fiber orientation, the direction is determined by a particular colour assigned for each axis i.e. red for X, green for Y and blue for Z axes) we would have to measure diffusion along thousands of axes, which is not practical. To simplify this issue, the concept of diffusion tensor was introduced in the early 1990s.
In this model, measurements along different axes are fitted to a 3D ellipsoid (Fig. 2.25 A). The properties of the 3D ellipsoid, namely, the length of the longest, middle, and shortest axes (called eigenvalues, $\lambda_1$, $\lambda_2$, and $\lambda_3$) and their orientations (called eigenvectors, $v_1$, $v_2$, and $v_3$) can be defined by six parameters (Fig. 2.25 B). Therefore, ADC measurements along six axes are enough to calculate the ellipsoid. To convert the measurement results (more than six ADC) to these six parameters, a $3 \times 3$ symmetric matrix called tensor is used, hence the name "diffusion tensor imaging." Once these six parameters are obtained at each pixel, several interesting contrasts can be generated. For example, we can measure the degree of diffusion anisotropy by using a measurement of difference among the three eigenvalues: $(\lambda_1 - \lambda_2)^2 + (\lambda_1 - \lambda_3)^2 + (\lambda_2 - \lambda_3)^2$. If diffusion is isotropic ($\lambda_1 = \lambda_2 = \lambda_3$), this measure becomes 0. Large numbers indicate high diffusion anisotropy. One of the most widely used metrics of diffusion anisotropy is "fractional anisotropy (FA)," which is (Pierpaoli and Basser, 1996):

$$FA = \frac{1}{2} \sqrt{\frac{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_3 - \lambda_1)^2}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}$$

This is a very convenient index because it is scaled from 0 (isotropic) to 1 (anisotropic) (Fig. 2.25 D). After a diffusion ellipsoid is determined, the information can be reduced to a vector of the longest axis (eigenvector $v_1$), which we assume indicates the fiber orientation (Fig. 2.25 C). Because it is very difficult to visualize 3D vectors, we usually convert this information to a color space (Fig. 2.25 F) and generate a color-coded orientation map (Fig. 2.25 E) (Makris et al., 1997; Pajevic and Pierpaoli, 1999).
**Fig. 2.25:** The Principal of DTI and Contrast Generation: From diffusion measurements along multiple axes (A), the shape and the orientation of a “diffusion ellipsoid” is estimated (B) this ellipsoid represents what an ink stain would be if ink were dropped within the pixel. An anisotropy map (D) can be created from the shape, in which dark regions are isotropic (spherical) and the bright regions are anisotropic (elongated). From the estimated ellipsoid (B), the orientation of the longest axis can be found (C), which is assumed to represent the local fiber orientation. This orientation information is converted to a color (F) at each pixel. By combining the intensity of the anisotropy map (D) and the color (F), a color-coded orientation map is created (E) (Adapted from Mori, 2006) (adapted from mori and zhang, 2006).

DTI based FA and MD values are the most sensitive parameters to change in membrane integrity affecting intracellular and extracellular water volume. The increase or decrease in the FA values reflects the integrity/the status of the myelin sheath around the axon under different condition of myelination, unmyelination and dysmyelination. On the other hand the brain MD changes are a function of intracellular-extracellular water homeostatis under different condition. The increase in MD values might be due to increased interstitial space, which might be due to reduced neural or glial cell packing or cell size, or decreased water exchange rate between the intra- and extracellular...
compartments, whereas the increase in the MD values might be due to influx of water from fast extracellular diffusion compartment to slow diffusion intracellular compartment.